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Analytical Methods

Bioavailability of calcium and its absorption inhibitors in raw and cooked green leafy vegetables commonly consumed in India – An in vitro study

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ABSTRACT

The objectives of this research were to assess the bioavailability of calcium using equilibrium dialysis after simulated gastric digestion method in 20 commonly consumed green leafy vegetables (GLVs) from the typical Indian diet, provide data on the content of calcium absorption inhibitors, like oxalate, phytate, tannin and dietary fibres, and evaluate the inhibitory effect of these compounds on calcium bioavailability in raw and cooked GLVs. Cooking did not affect significantly calcium bioavailability in any GLVs. *Sesbania grandiflora* had a very high content of total oxalates, tannins and dietary fibers, which reduced calcium bioavailability. Calcium content was determined by atomic absorption spectroscopy, oxalate by titrimetry, phytate and tannin by colorimetric and dietary fibres by an enzymatic gravimetric method. *Chenopodium album*, *Alternanthera philoxeroides* and *Centella asiatica*, with lower total calcium content, had nearly twice as much bioavailable calcium than other GLVs, because of low fibres, oxalate, phytate and tannin content.

1. Introduction

Calcium, the most abundant mineral in the body, is found in some foods, added to others, available as a nutritional supplement, and present in some medicines. The body's calcium supply is stored in the bones and teeth where it supports their structure and function. Bone undergoes continuous remodelling, with constant resorption and deposition of calcium into new bone. The balance between bone resorption and deposition changes with age. Bone formation exceeds resorption in periods of growth in children and adolescents, whereas in early and middle adulthood both processes are relatively equal. In ageing adults, particularly among postmenopausal women, bone breakdown exceeds formation, resulting in bone loss that increases the risk of osteoporosis over time (IOM, 2010). Calcium (Ca) is an essential nutrient for plants and animals, with key structural and signalling roles, and its deficiency in plants can result in poor biotic and abiotic stress tolerance, reduced crop quality and yield. Likewise, inadequate calcium (Ca) intake and poor absorption of Ca in human are among several risk factors for osteoporosis and some other diseases (e.g. rickets, hypertension and colorectal cancer) (Centeno, de Barboza, Marchionatti, Rodriguez, & de Talamoni, 2009).

Osteoporosis is a condition that mainly affects older people and is linked to enhanced decalcification and demineralization of bones. Most, but not all, studies show that increasing Ca intake in later life decreases the occurrence of osteoporotic fractures and it is universally accepted that sustaining the recommended dietary intake (RDI) of Ca throughout life is beneficial for health later in years (Michaelsson, 2009).

In most developing countries, vegetables are the most reliable and affordable source of minerals and vitamins for families (Mosha, Gaga, Pace, Laswai, & Mtebe, 1995). In India, most of the people adopt a vegetarian lifestyle. Plant-food-based diets are rich in bioactive compounds, which are believed to be advantageous for the prevention of non-communicable chronic diseases, such as cancer, diabetes mellitus, etc. Green leafy vegetables occupy an important place among the food crops as these provide adequate amounts of many vitamins and minerals for humans. They are rich source of carotene, ascorbic acid, riboflavin, folic acid and minerals like calcium, iron and phosphorous. The contribution of minerals and vitamins from vegetable in human nutrition is, however, limited due to the presence of anti-nutritional factors, which render some of the nutrients unavailable for absorption. The most common anti-nutritional factors present in GLVs that decrease the

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bioavailability of some minerals, especially calcium, are oxalate, phytate, tannin and dietary fibres (DF) (Mosha et al., 1995).

The bioavailability of minerals has generated increasing interest in the field of nutrition. Bioavailability should be determined by in vivo measurements, ideally in humans. However, such studies are difficult, expensive, and provide limited data (Camara, Amaro, Barbera, & Clemente, 2005). While animal assays are less expensive, they are somewhat limited by uncertainties with regard to differences in metabolism between animals and humans. As an alternative to human and animal studies in vivo are measures made using simple, rapid and inexpensive methods in vitro (Luten et al., 1996).

In vitro estimation of the bioavailability of minerals from foods involves the simulation of gastrointestinal digestion and measurement of the mineral soluble fraction or the mineral fraction that dialyses across a semi-permeable membrane of a certain pore size. These methods are widely used because of their good correlation with in vivo studies. In vitro methods have been used to estimate the bioavailability of minerals from different foods and also from dishes and composite diets (Camara et al., 2005).

In the present study, 20 GLVs commonly consumed in India were selected for determining their calcium content and bioavailability in vitro. Various factors that may influence bioavailability of calcium, namely oxalate, phytate, tannin and dietary fibre were also quantified. Bioavailability of calcium was correlated with the concentration of these inhibitory factors since there is very little information in the literature about the content of oxalate, phytate, tannin and dietary fibres in raw and cooked GLVs. Therefore, one of the objectives of this study was to analyse the effects of cooking on the content of oxalate, phytate, tannin and dietary fibre. These data form a basis for better food selection and, consequently, improved nutritional advice for local populations in India as well as potentially preventing the onset of fluorosis.

2. Materials and methods

2.1. Materials

Locally grown and commonly consumed fresh GLVs were purchased from different markets in Dindigul District, Tamil Nadu (India) and healthy, disease-free, edible parts selected for this study. All chemicals used in the experiments were of analytical (AR) grade and were obtained from Sigma Aldrich India Ltd., Mumbai, India. Millipore – MilliQ distilled water was employed during the complete study.

2.2. Preparation of the samples

Twenty samples of GLVs collected were *Acalypha indica*, *Allmania nodiflora*, *Alternanthera dentate*, *Alternanthera lehmannii*, *Alternanthera philoxeroides*, *Alternanthera sessilis*, *Amaranthus blitum*, *Amaranthus dubius*, *Amaranthus polygonoides*, *Amaranthus spinosus*, *Basella alba*, *Centella asiatica*, *Chenopodium album*, *Hibiscus sabdariffa (Linn)*, *Marsilea villosa*, *Moringa oleifera*, *Pisonia alba*, *Sesbania grandiflora*, *Solanum nigrum* and *Trigonella foenum-graecum* were selected. They were cleaned and washed with Millipore water after separating the non-edible portion. The thoroughly drained greens were cut into 1 cm pieces and they were divided into two parts. One part was cooked using Millipore – MilliQ distilled water in microwave oven until the water was evaporated and marked. The cooked and fresh samples were dried in glass dishes in a hot air oven at 50 ± 5 °C. The dried samples were ground to fine powder and stored in airtight containers. The dried samples were used for the estimation of total, soluble and bioavailable calcium as well as oxalate, phytate, tannin and dietary fibres.

2.3. Determination of total calcium content

Finely ground GLV samples were ashed in a muffle furnace at 550 °C for 10 h and the ash was dissolved in conc. HCl. Calcium content was determined by atomic absorption spectrometer (Perkin – Elmer A Analyst 100). Instrumental conditions: wavelength = 422.7 nm, slit = 0.7 nm, recommended flame = air–acetylene, oxidising (lean, blue) and nebulizer = spoiler. Lanthanum chloride (1%) was added to the mineral solution to avoid interference from phosphate. Calibration of the instrument was performed using commercial standards. All measurements were carried out using standard flame operating conditions, as recommended by the manufacturer.

2.4. Evaluation of calcium bioavailability by in vitro simulated gastrointestinal method

Bioavailability of calcium from selected GLV samples were determined using an in vitro method described by Luten et al. (1996), involving simulated gastrointestinal digestion (Fig. S1; supporting information) with suitable modifications. All finely ground GLVs were subjected to simulated gastric digestion by incubation with pepsin (pH 2.0) at 37 °C for 2 h. Titratable acidity was measured in an aliquot of the gastric digest, by adjusting the pH to 7.5 with 0.1 mol L^{-1} NaOH in the presence of pancreatin–bile extract mixture (1 L of 0.1 mol L^{-1} sodium bicarbonate containing 4 g pancreatin + 25 g bile extract). Titratable acidity was defined as the amount of 0.1 mol L^{-1} NaOH required to attain a pH of 7.5. To simulate intestinal digestion, segments of dialysis tubing (Molecular mass cut off between 12,000 and 14,000 Da) containing 25 ml aliquots of sodium bicarbonate solution, being equivalent in moles to the NaOH needed to neutralize the gastric digest (titratable acidity) determined as above, were placed in Erlenmeyer flasks containing the gastric digest and were incubated at 37 °C with shaking for 30 min or longer until the pH of the digest reached 5.0. Five millilitres of the pancreatin–bile extract mixture was then added and incubation was continued for 2 h or longer until the pH of the digest reached 7.0. At the end of simulated gastro-intestinal digestion, calcium present in the dialyzate was analysed by atomic absorption spectrometry. The dialyzable portion of the total calcium present in the sample (expressed as percent) represented the bioavailable calcium.

Bioavailability (%) was calculated as follows: bioavailability (%) = $100 \times D/T$, where, D is the calcium content in the dialyzable portion for the bioavailable fraction (mg calcium/100 g GLVs), and T is the total calcium content (mg calcium/100 g GLVs).

2.5. Determination of inhibitory factors

2.5.1. Determination of oxalate

The GLVs were analysed for total oxalates and soluble oxalates by precipitation with calcium oxalate from deproteinized extract and subsequent titration with potassium permanganate (Association of Official Analytical Chemists – AOAC, 2000). For total oxalate determination, 5 g sample and 200 ml distilled water are placed in a 600-ml Berzelius beaker, and this mixture was stirred for 15 min. Then 100 ml distilled water and 55 ml of 6 mol L^{-1} HCl were added and boiled with reflux for another 15 min; after this time, the mixture was allowed to cool. Then it was adjusted to 500 ml with distilled water and left overnight. Then the mixture was filtered. The determination of oxalate was carried out by precipitation as calcium oxalate (AOAC, 2000). To 25 ml of filtrate, 5 ml tungstophosphoric acid solution (prepared by mixing 2.5 g $Na_2WO_4 \cdot H_2O$ to 4 ml H_3PO_4 with concentration 1.2 mol L^{-1}, and diluting to 100 ml) was added and left to stand for at least 5 h. After this period of time, the mixture was filtered, an aliquot of 20 ml was taken

and the pH was adjusted to 4.0–4.5 with concentrated ammonia. To this solution, 5 ml of a pH 4.5 acetate buffer solution, containing 0.45 mol L^{-1} $CaCl_2$, was added and left overnight in order to precipitate oxalate ions. Afterwards, the precipitate was centrifuged at 1,700 rpm and washed with a 0.9 mol L^{-1} acetic acid solution, and 5 ml of 0.45 mol L^{-1} $CaCl_2$. Finally, the precipitate was dissolved in 5 ml of 1:1 sulphuric acid and titrated with a 0.002 mol L^{-1} $KMnO_4$ standard solution.

For water soluble oxalate determination, 5 g sample and 200 ml distilled water were placed in a 600-ml Berzelius beaker, and this mixture was boiled for 15 min. After this time, the mixture was allowed to cool. Then it was adjusted to 500 ml with distilled water and left overnight. Then the mixture was filtered and oxalate determination was carried out using the same procedure described for total oxalate.

2.5.2. Determination of phytate

Phytate content was determined as described by Wheeler and Ferrel (1971). Phytic acid was extracted from 1 g GLV sample with 50 ml of 3% tri chloro acetic acid by shaking at room temperature followed by high speed centrifugation. The phytic acid in supernatant was precipitated as ferric phytate by adding excess ferric chloride and centrifuged. The ferric phytate was converted to ferric hydroxide with a few ml of water and 3 ml of 1.5 mol L^{-1} NaOH, and then the precipitated ferric hydroxide was dissolved with 3.2 mol L^{-1} HNO_3. The iron content present in the sample was estimated by the addition of 1.5 mol L^{-1} KSCN and read colorimetrically at 480 nm using UV–vis spectrophotometer (Perkin Elmer Lambda 35). The phytate phosphorus was calculated from the iron results assuming a 4:6 iron:phosphorus molecular ratio. The phytate was estimated by multiplying the amount of phytate phosphorus by the factor 3.55 based on the empirical formula $C_6P_6O_{24}H_{18}$.

2.5.3. Determination of tannin

Tannin compounds reduce phosphotungstomolybdic acid in alkaline solution to produce a highly coloured blue solution, the intensity of colour is proportional to the amount of tannins. 0.5 g of powdered GLV samples was weighed, transferred to a 250 ml conical flask and 75 ml water added. The flask was heated gently and boiled for 30 min before the mixture was centrifuged at 2000 rpm for 20 min and the supernatant collected. 5 ml of Folin–Denis reagent was added to the supernatant and 10 ml of Na_2CO_3. Colour intensity was measured in a UV–vis spectrophotometer (Perkin Elmer Lambda 35) at 700 nm (Schanderl, 1970).

2.5.4. Determination of total (TDF), insoluble (IDF) and soluble (SDF) dietary fibre

Moisture and fat free GLV samples were analysed for their TDF, IDF and SDF contents by an enzymatic and gravimetric method (Prosky, Asp, Schwizer, De Vries, & Furda, 1988), using a TDF-100 kit obtained from Sigma Aldrich chemical company (Mumbai, India). In the parallel with the samples, blank and reference controls were analysed in duplicate for comparison.

IDF determination was carried out as follows. Pre-weighed crucibles containing celite, previously washed with water, were used to filter the enzyme digest; this was washed with two 10 ml portions of water. The filtrate and the water washings were saved for SDF determination. The residue was determined following sample digestion with thermo-stable α-amylase at pH 6.0 for 30 min at 100 °C and allowed to cool. The pH was adjusted to 7.5 and the sample was incubated with protease for 30 min at 60 °C. After cooling, the sample was adjusted to pH 4.5 and incubated with amyloglucosidase at 60 °C for 30 min. All incubations were carried out in a boiling water bath with continuous shaking. A 250-ml volume of 95% ethanol preheated to 60 °C was added and the sample was allowed to precipitate at room temperature for 60 min. Pre-weighed crucibles, containing celite previously washed with 78% ethanol, were used to filter the enzyme digest. The residue was washed with 78% ethanol, 95% ethanol and acetone, then dried in an air oven, cooled in desiccators and weighed for IDF determination. Determination of protein, ash and calculation of SDF were carried as described by Prosky et al. (1988).

SDF was determined in the combined filtrate and washings from the IDF procedure as described above. This solution was adjusted to 100 g with water and precipitated with 95% ethanol preheated to 60 °C. After filtration through a pre-weighed crucible containing celite, the residue was washed successively with 78% ethanol, 95% ethanol and acetone. The crucible was dried overnight in a 105 °C air oven. Determination of protein, ash and calculation of SDF were carried out as described by Prosky et al. (1988). The TDF, which was calculated as the sum of IDF and SDF.

2.6. Quality control

To eliminate the risk of contamination, all glassware used was immersed in 10% (v/v) nitric acid for 24 h, and washed with Millipore – MilliQ distilled water before use. For calcium, oxalate, phytate and tannin, recovery was found to be 98.2%, 96.8%, 97.4% and 96.6%, respectively. For dietary fibres, along with samples, blank and reference controls were analysed in duplicate for comparison. Accuracy and reproducibility of the method for calcium, oxalate, phytate and tannin were checked by adding two known concentrations of calcium, oxalic acid, phytic acid and tannic acid (10 and 20 mg L^{-1}) to selected GLVs. Five replicates of each GLV were analysed on three different days.

2.7. Statistical analysis

All determinations were done in five replicates, and the average values, mean and standard deviation are reported. The data were also analysed statistically by multiple regression analysis, to determine the extent of modulation of calcium bioavailability by oxalate, phytate, tannin and dietary fibre using the statistical software SPSS 16.

3. Results and discussion

The analysed GLVs were found to be rich sources of calcium. The calcium content of the GLV varied widely, ranging from 453.2 mg/100 g in *A. lehmannii* to 1083.7 mg/100 g in *A. polygonoides*. Three GLVs, *A. polygonoides*, *P. alba* and *A. spinosus* were found to have exceptionally high calcium content of 1083.7, 1062.4 and 1035.3 mg/100 g, respectively. *A. philoxeroides*, *C. asiatica*, *A. blitum* and *A. lehmannii* were found to have lower levels of calcium (498.1, 493.9, 472.8 and 453.2 mg/100 g, respectively) while the rest of the GLVs contained calcium in the range of 521.3–953.4 mg/100 g (Table 1).

Bioavailable calcium was found to be highest in *A. polygonoides* (239.6 mg/100 g), followed by *A. sessilis* and *A. nodiflora*, which were 209.5 and 179.5 mg/100 g, respectively. *A. lehmannii* had the least bioavailable calcium of 95.8 mg/100 g, while in the rest of the GLVs it varied from 98.3 to 176.7 mg/100 g dry weight. The percentage of bioavailable calcium content also varied considerably from 14.8% in *S. grandiflora* to 33.7% in *C. album* (Table 1). Solubility and bioavailable percentages ranged from 21.0% (*A. spinosus*) to 69.6% (*C. album*), and from 14.8% (*S. grandiflora*) to 32.7% (*C. album*), respectively (Table 1). These values are higher than those reported by Gupta, Jyothi Lakshmi, and Prakash (2006), Gupta, Jyothi Lakshmi, and Manjunath (2005) and Kala and Prakash (2004). Solubility and bioavailability, expressed as

Table 1
Total, soluble and bioavailable calcium content in selected GLVs.

GLVs	Total		Soluble		Soluble (%)		Bioavailable		Bioavailable (%)	
	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked
Acalypha indica	650.5 ± 1.2	654.6 ± 1.7	362.7 ± 1.7	365.8 ± 1.6	55.8	55.9	169.8 ± 1.2	170.6 ± 1.2	26.1	26.1
Allmania nodiflora	877.1 ± 1.6	883.3 ± 1.1	260.4 ± 1.6	264.2 ± 1.8	29.7	29.9	179.5 ± 0.9	180.4 ± 1.0	20.5	20.4
Alternanthera dentata	582.8 ± 1.0	584.2 ± 0.9	261.0 ± 1.8	261.9 ± 1.7	44.8	44.8	161.8 ± 1.1	165.6 ± 2.0	27.8	28.3
Alternanthera lehmannii	453.2 ± 2.2	461.2 ± 2.3	226.7 ± 0.9	228.7 ± 1.1	50.0	49.6	95.8 ± 0.2	99.5 ± 0.9	21.1	21.6
Alternanthera philoxeroides	498.1 ± 2.3	503.1 ± 1.4	264.9 ± 2.9	266.0 ± 1.8	53.2	52.9	161.3 ± 1.9	162.5 ± 2.5	32.4	32.3
Alternanthera sessilis	918.1 ± 9.4	925.2 ± 1.0	364.6 ± 1.2	366.0 ± 1.3	39.7	39.6	209.5 ± 1.1	212.8 ± 1.6	22.8	23.0
Amaranthus blitum	472.8 ± 0.7	475.5 ± 0.2	217.5 ± 0.4	218.2 ± 0.5	46.0	45.9	110.6 ± 0.5	111.1 ± 0.7	23.4	23.4
Amaranthus dubius	835.4 ± 2.1	840.2 ± 0.2	241.8 ± 1.8	245.4 ± 0.9	28.9	29.2	163.6 ± 1.9	165.6 ± 1.5	19.6	19.7
Amaranthus polygonoides	1083.7 ± 1.6	1090.9 ± 1.5	557.9 ± 3.0	562.0 ± 2.7	51.5	51.1	239.6 ± 1.6	242.1 ± 2.6	22.1	22.2
Amaranthus spinosus	1035.3 ± 2.4	1038.7 ± 2.2	217.8 ± 1.2	220.0 ± 1.2	21.0	21.2	175.0 ± 1.2	176.1 ± 0.8	16.9	17.0
Basella alba	551.4 ± 2.4	557.1 ± 3.1	227.4 ± 2.0	224.7 ± 2.1	41.2	40.3	152.8 ± 1.8	154.9 ± 2.0	27.7	27.8
Centella asiatica	493.9 ± 3.0	498.4 ± 1.7	264.3 ± 1.2	268.9 ± 0.9	53.5	53.9	158.7 ± 0.9	164.1 ± 1.9	32.1	32.9
Chenopodium album	539.9 ± 2.0	545.7 ± 1.8	375.8 ± 1.3	377.5 ± 1.0	69.6	69.2	176.7 ± 2.2	180.8 ± 2.2	32.7	33.1
Hibiscus sabdariffa (Linn)	554.9 ± 3.1	560.6 ± 3.0	251.1 ± 6.0	253.8 ± 1.2	45.3	45.3	163.3 ± 1.4	165.6 ± 0.5	29.4	29.5
Marsilea villosa	815.1 ± 2.3	822.3 ± 1.5	449.9 ± 2.7	451.3 ± 2.9	55.2	54.9	124.6 ± 1.2	126.2 ± 1.4	15.3	15.3
Moringa oleifera	769.5 ± 1.8	773.2 ± 1.7	469.8 ± 1.2	472.9 ± 0.7	61.1	61.2	152.3 ± 6.5	157.6 ± 0.7	19.8	20.4
Pisonia alba	1062.4 ± 2.4	1069.2 ± 1.8	455.8 ± 2.2	460.1 ± 0.8	42.9	43.0	173.6 ± 1.4	174.5 ± 1.0	16.3	16.3
Sesbania grandiflora	664.6 ± 2.3	666.3 ± 2.0	335.6 ± 2.8	337.0 ± 2.5	50.5	50.6	98.3 ± 2.6	102.1 ± 0.6	14.8	15.3
Solanum nigrum	953.4 ± 2.1	955.4 ± 2.1	434.9 ± 2.0	435.7 ± 1.3	45.6	45.6	147.7 ± 1.4	150.0 ± 1.3	15.5	15.7
Trigonella foenum-graecum	521.3 ± 2.4	522.3 ± 0.8	209.3 ± 0.5	209.8 ± 0.8	40.1	40.2	110.4 ± 0.1	111.6 ± 0.4	21.2	21.3

Values (mg/100 g) are means ± SEM of five independent determinations.

Table 2
Total, soluble and insoluble oxalate content in selected GLVs.

GLVs	Total		Soluble		Soluble (%)		Insoluble		Insoluble (%)	
	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked
Acalypha indica	644.9 ± 1.9	408.5 ± 2.3	336.1 ± 1.4	149.8 ± 2.1	52.1	36.7	308.8 ± 1.6	258.7 ± 1.5	47.9	63.3
Allmania nodiflora	855.5 ± 1.1	553.9 ± 2.4	476.2 ± 1.4	257.6 ± 2.1	55.7	46.5	379.3 ± 1.4	296.3 ± 3.3	44.3	53.5
Alternanthera dentata	756.3 ± 0.5	560.6 ± 3.0	394.5 ± 0.5	192.5 ± 1.1	52.2	34.3	361.8 ± 0.4	368.1 ± 3.4	47.8	65.7
Alternanthera lehmannii	1096.6 ± 1.3	862.4 ± 1.6	793.9 ± 1.7	377.3 ± 2.2	72.4	43.8	302.7 ± 2.8	485.1 ± 1.5	27.6	56.2
Alternanthera philoxeroides	584.5 ± 1.5	401.2 ± 3.1	217.1 ± 1.2	156.9 ± 2.5	37.1	39.1	367.4 ± 2.2	244.3 ± 3.6	62.9	60.9
Alternanthera sessilis	619.3 ± 0.6	350.8 ± 2.2	201.7 ± 0.5	103.4 ± 1.1	32.6	29.5	417.6 ± 0.4	247.3 ± 2.9	67.4	70.5
Amaranthus blitum	568.8 ± 0.3	282.4 ± 0.6	201.0 ± 1.1	101.6 ± 2.5	35.3	36.0	367.8 ± 0.4	180.9 ± 0.5	64.7	64.0
Amaranthus dubius	678.2 ± 1.0	437.5 ± 2.6	321.9 ± 0.7	202.1 ± 1.6	47.5	46.2	356.3 ± 1.0	235.4 ± 3.1	52.5	53.8
Amaranthus polygonoides	604.6 ± 0.6	466.7 ± 2.9	298.6 ± 0.8	205.5 ± 1.2	49.4	44.0	306.1 ± 1.2	261.2 ± 2.6	50.6	56.0
Amaranthus spinosus	1098.3 ± 1.3	846.7 ± 2.7	660.9 ± 1.8	419.9 ± 1.6	60.2	49.6	437.4 ± 2.6	426.7 ± 3.1	39.8	50.4
Basella alba	593.1 ± 1.1	418.7 ± 2.7	234.9 ± 0.8	185.4 ± 2.6	39.6	44.3	358.2 ± 1.4	233.3 ± 2.2	60.4	55.7
Centella asiatica	849.0 ± 1.1	634.6 ± 3.0	258.7 ± 1.2	251.3 ± 1.8	30.5	39.6	590.3 ± 1.6	383.3 ± 3.2	69.5	60.4
Chenopodium album	744.1 ± 1.5	383.7 ± 3.2	243.6 ± 0.8	189.2 ± 1.1	32.7	49.3	500.5 ± 1.8	194.5 ± 3.8	67.3	50.7
Hibiscus sabdariffa (Linn)	1001.9 ± 1.2	709.0 ± 3.4	451.9 ± 1.4	322.8 ± 2.4	45.1	45.5	550.0 ± 2.4	386.2 ± 3.4	54.9	54.5
Marsilea villosa	723.5 ± 0.8	454.9 ± 3.3	408.5 ± 1.2	259.1 ± 2.1	56.5	57.0	315.0 ± 1.5	195.8 ± 3.4	43.5	43.0
Moringa oleifera	795.6 ± 0.8	567.8 ± 2.3	396.3 ± 1.7	368.7 ± 2.3	49.8	64.9	399.4 ± 1.0	199.1 ± 2.8	50.2	35.1
Pisonia alba	814.4 ± 0.6	639.7 ± 3.2	386.6 ± 0.7	366.2 ± 1.6	47.5	57.2	427.9 ± 0.5	273.5 ± 3.1	52.5	42.8
Sesbania grandiflora	1199.2 ± 0.8	970.5 ± 1.2	648.4 ± 2.0	490.9 ± 3.4	54.1	50.6	550.8 ± 2.8	479.6 ± 2.3	45.9	49.4
Solanum nigrum	776.2 ± 1.4	567.1 ± 3.4	337.5 ± 1.6	328.5 ± 2.2	43.5	57.9	438.7 ± 2.3	238.6 ± 3.4	56.5	42.1
Trigonella foenum-graecum	682.5 ± 2.7	366.1 ± 2.3	282.9 ± 2.6	207.8 ± 0.4	41.4	56.8	399.6 ± 0.4	158.3 ± 0.3	58.6	43.2

Values (mg/100 g) are means ± SEM of five independent determinations.

mg/100 g, showed a significant ($p < 0.01$) correlation with Ca content – soluble Ca ($r = 0.60$) and bioavailable Ca ($r = 0.58$).

GLVs are an important part of the diet of Indians, especially people of the locality of the study, who regularly consume such foods. Although rich in calcium, some GLVs have low calcium bioavailability and a high content of inhibitory factors, such as oxalates, phytates, tannins and DF, which bind calcium rendering it insoluble and, therefore, unavailable to the body.

Oxalic acid is one of the anti-nutritional factors, which are widely distributed in plant foods. Oxalic acid is known to interfere with calcium absorption by forming insoluble salts of calcium. *S. grandiflora*, *A. spinosus*, *A. lehmannii* and *H. sabdariffa* were found to have high total oxalate values of 1199.2, 1098.3, 1096.6 and 1001.9 mg/100 g, respectively (Table 2). The rest of the GLVs had less than 1000 mg/100 g of total oxalate content, in the range of 568.8 to 855.5 mg/100 g. *A. blitum* and *A. philoxeroides* had very low values of oxalate contents, 568.8 and 584.5 mg/100 g, respectively. Oxalic acid values of some of the GLVs analysed in this study were also found to be in the same range as reported in the literature (Gupta et al., 2006, 2005; Kumari, Gupta, Jyothi Lakshmi, & Prakash, 2004). Soluble oxalates varied from 201.0 to 793.3 mg/100 g in the analysed GLVs. Statistically significant differences were found in the total and soluble oxalate content among the GLV ($p < 0.01$). The soluble oxalate content of GLVs also differed considerably with *A. lehmannii* having the most soluble oxalates (72.4%) and *C. album* having the least (32.7%). The percentages of soluble oxalate were similar to those reported by Moreau and Savage (2009), Radek and Savage (2008), Judprasong, Charoenkiatkul, Sungpuag, Vasanachitt, and Nakjamanong (2006) and Gupta et al. (2005).

Phytate is a hexaphosphate of inositol and binds calcium and iron rendering these unavailable (Gupta et al., 2006). Phytate

Table 3
Phytate and tannin content in selected GLVs.

GLVs	Phytate		Tannin	
	Raw	Cooked	Raw	Cooked
Acalypha indica	61.8 ± 0.9	59.4 ± 0.4	94.5 ± 0.3	95.0 ± 0.9
Allmania nodiflora	112.1 ± 1.0	114.0 ± 0.2	256.6 ± 1.3	256.6 ± 0.3
Alternanthera dentata	68.8 ± 0.2	69.4 ± 1.5	231.7 ± 0.4	233.0 ± 0.6
Alternanthera lehmannii	124.9 ± 0.9	122.7 ± 0.4	196.6 ± 0.2	199.2 ± 1.3
Alternanthera philoxeroides	68.8 ± 0.5	67.1 ± 0.8	180.9 ± 0.8	177.8 ± 1.6
Alternanthera sessilis	57.1 ± 0.3	56.6 ± 0.4	229.1 ± 0.5	228.9 ± 0.7
Amaranthus blitum	61.9 ± 0.5	63.0 ± 0.8	204.2 ± 0.8	203.2 ± 1.8
Amaranthus dubius	67.1 ± 0.2	67.1 ± 0.4	224.5 ± 1.4	220.0 ± 0.9
Amaranthus polygonoides	42.4 ± 0.3	40.7 ± 0.3	214.6 ± 1.3	215.2 ± 0.6
Amaranthus spinosus	161.1 ± 0.4	159.1 ± 0.8	129.0 ± 1.1	127.9 ± 1.3
Basella alba	41.7 ± 0.4	42.6 ± 0.5	221.3 ± 0.6	225.1 ± 1.1
Centella asiatica	24.5 ± 1.3	24.0 ± 0.7	224.6 ± 0.5	225.0 ± 0.6
Chenopodium album	73.5 ± 0.4	72.9 ± 0.8	86.5 ± 0.5	84.4 ± 1.1
Hibiscus sabdariffa (Linn)	121.8 ± 0.4	122.2 ± 0.4	296.6 ± 1.2	300.6 ± 0.6
Marsilea villosa	46.1 ± 0.2	45.8 ± 0.3	227.6 ± 0.5	222.5 ± 0.9
Moringa oleifera	58.6 ± 0.2	57.3 ± 0.6	263.3 ± 1.6	266.8 ± 1.2
Pisonia alba	74.6 ± 0.3	74.0 ± 1.1	271.3 ± 0.5	273.1 ± 0.8
Sesbania grandiflora	135.2 ± 1.3	137.4 ± 0.5	424.7 ± 1.6	416.1 ± 1.3
Solanum nigrum	58.8 ± 0.7	59.9 ± 0.6	355.5 ± 0.6	360.1 ± 0.6
Trigonella foenum-graecum	72.9 ± 0.7	71.4 ± 1.0	176.4 ± 0.5	175.3 ± 1.6

Values (mg/100 g) are means ± SEM of five independent determinations.

contents of the analysed GLVs were found to be in the range of 24.5–161.1 mg/100 g of dry weight sample (Table 3). These values are considerably higher than those reported by other workers (Gupta et al., 2006, 2005) indicating that the higher phytate content would counter the higher bioavailability of minerals due to the formation of insoluble complexes.

The tannin content of the GLVs varied widely, and the values are shown in Table 3. Tannin was highest in *S. grandiflora* (424.7 mg/100 g) followed by *S. nigrum* (355.5 mg/100 g) and *H. sabdariffa (Linn)* (296.6 mg/100 g). The least tannin (86.5 mg/100 g) was registered by *C. album*. The tannin content of selected GLVs were in the same order of magnitude as those reported by earlier works (Gupta et al., 2006, 2005; Sotelo, Gonzalez-Osnaya, Sanchez-Chinchillas, & Trejo, 2010).

Values of SDF, IDF and TDF were calculated as per the method reported by Prosky et al. (1988) and the results are shown in Table 4. The contents of IDF in the GLV were found to be in the range of 26.1 g/100 g in *A. spinosus* to 69.6 g/100 g in *P. alba*. *B. alba* and *S. grandiflora* had SDF content of 6.4 and 7.9 g/100 g respectively, while in the rest of the GLVs it varied from 10.6 to 28.0 g/100 g. Significant differences were found between TDF and IDF content among the GLVs ($p < 0.01$). *A. spinosus* had a low TDF content of 26.1 g/100 g and *P. alba* had high TDF content of 69.6 g/100 g. These GLVs were found to have lower TDF contents when compared to the reports of other researchers (Gupta et al., 2006, 2005; Kumari et al., 2004; Kala & Prakash, 2004).

Components of GLVs, like oxalate, phytate, tannin and dietary fiber, negatively affect Ca bioavailability (Gupta et al., 2006). The presence of these inhibitors could explain the relatively low bioavailable percentage of calcium obtained for *S. grandiflora* 14.8% and 16.9% for *A. spinosus*, respectively, even though their total calcium contents were relatively high (Table 1). *A. spinosus* had high calcium content, but also contained high amounts of oxalate that affect calcium bioavailability. This could be a reason for the lowest percentages of soluble calcium (21.0%) in *A. spinosus* and the lowest percentage of bioavailable calcium (16.9%) despite having relatively higher total calcium content (1035.3 mg/100 g) compared with the other GLVs (Table. 1). Similar results were reported by Gupta et al. (2006) who indicated that, among the foods analysed, *Digera arvensis forsk* had the lowest bioavailable calcium (4%) and the highest oxalate content (1198 mg/100 g). *A. spinosus* also contained a high concentration of phytate (161.1 mg/100 g) and phytate content is recognised to have a negative correlation with mineral bioavailability.

Comparing the calcium contents of the cooked GLVs, *A. polygonoides* had the highest (1090.9 mg/100 g) and *A. lehmannii* contained the lowest calcium content 461.2 mg/100 g. The higher mineral content in the leaves could be due to the fact that chicken and cattle manure are commonly used as fertilizers for the crops by the local formers. Animal manure contains significant amounts of nutrients (nitrogen, phosphorus, calcium, potassium, magnesium, copper and zinc), which are easily absorbed by plants (Schonfeldt & Pretorius, 2011).

Table 4
Total, soluble and insoluble dietary fibres in selected GLVs.

GLVs	IDF		SDF		TDF		SDF (%)	
	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked
Acalypha indica	39.0 ± 0.4	44.7 ± 0.7	10.6 ± 0.4	11.3 ± 0.1	49.6 ± 0.8	56.0 ± 0.8	21.3	20.2
Allmania nodiflora	48.5 ± 0.2	50.8 ± 0.5	18.6 ± 0.6	20.0 ± 0.2	67.2 ± 0.8	70.8 ± 0.7	27.8	28.2
Alternanthera dentata	28.5 ± 0.4	33.2 ± 0.5	15.8 ± 0.4	15.9 ± 0.2	44.3 ± 0.8	49.1 ± 0.7	35.7	32.4
Alternanthera lehmannii	47.8 ± 0.5	51.9 ± 0.7	16.4 ± 0.4	17.2 ± 0.3	64.1 ± 0.8	69.1 ± 1.0	25.5	24.9
Alternanthera philoxeroides	44.0 ± 0.3	47.5 ± 0.8	20.0 ± 0.4	20.8 ± 0.4	64.0 ± 0.6	68.3 ± 1.2	31.2	30.5
Alternanthera sessilis	59.6 ± 0.9	62.6 ± 0.4	15.9 ± 0.4	16.8 ± 0.4	75.5 ± 1.3	79.4 ± 0.8	21.0	21.2
Amaranthus blitum	44.4 ± 0.4	48.6 ± 0.6	19.0 ± 0.3	20.8 ± 0.5	63.4 ± 0.8	69.4 ± 1.1	29.9	30.0
Amaranthus dubius	41.1 ± 0.2	44.7 ± 0.6	19.8 ± 0.5	21.3 ± 0.5	60.9 ± 0.7	66.0 ± 1.1	32.6	32.3
Amaranthus polygonoides	43.8 ± 0.3	48.6 ± 0.8	22.1 ± 0.3	23.1 ± 0.3	65.9 ± 0.6	71.7 ± 1.1	33.5	32.2
Amaranthus spinosus	26.1 ± 0.3	30.8 ± 0.4	15.2 ± 0.4	16.2 ± 0.3	41.3 ± 0.7	47.0 ± 0.7	36.9	34.5
Basella alba	56.7 ± 0.9	61.7 ± 0.4	6.4 ± 0.4	7.8 ± 0.3	63.1 ± 1.3	69.5 ± 0.7	10.1	11.2
Centella asiatica	59.6 ± 1.0	61.4 ± 0.9	14. 3 ± 0.5	15.8 ± 0.2	73.9 ± 1.5	77.2 ± 1.1	19.4	20.5
Chenopodium album	39.3 ± 1.0	43.2 ± 0.5	28.0 ± 0.3	29.9 ± 0.4	67.3 ± 1.2	73.1 ± 0.9	41.6	40.9
Hibiscus sabdariffa (Linn)	42.9 ± 0.6	46.4 ± 0.7	15.6 ± 0.5	16.6 ± 0.2	58.4 ± 1.1	63.0 ± 0.9	26.7	26.3
Marsilea villosa	39.3 ± 0.6	42.4 ± 0.7	21.1 ± 0.3	22.4 ± 0.3	60.3 ± 0.9	64.8 ± 1.0	34.9	34.6
Moringa oleifera	33.0 ± 0.3	36.1 ± 0.6	21.2 ± 0.3	22.8 ± 0.1	54.2 ± 0.6	58.9 ± 0.7	39.1	38.7
Pisonia alba	69.6 ± 0.7	73.0 ± 0.5	17.9 ± 0.3	19.1 ± 0.3	87.5 ± 0.9	92.1 ± 0.8	20.4	20.7
Sesbania grandiflora	45.6 ± 0.6	51.3 ± 0.5	7.9 ± 0.7	9.7 ± 0.7	53.5 ± 1.2	61.0 ± 1.2	14.7	15.8
Solanum nigrum	29.4 ± 0.8	32.5 ± 0.9	20.8 ± 0.5	21.7 ± 0.2	50.2 ± 1.3	54.2 ± 1.1	41.4	40.0
Trigonella foenum-graecum	41.7 ± 0.8	48.7 ± 0.4	27.4 ± 0.6	28.6 ± 0.2	69.1 ± 1.3	77.3 ± 0.6	39.6	37.0

Values (g/100 g) are means ± SEM of five independent determinations.

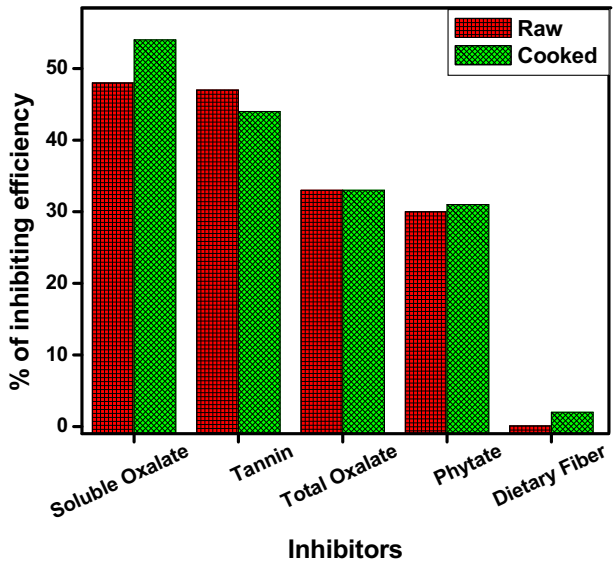

Fig. 1. Multiple regression analysis between percentage of bioavailable calcium and soluble oxalate, tannin, total oxalate, phytate and dietary fibre.

Results for oxalate in GLVs are presented in Table 2. The level of total oxalate in plant foods was found to inversely correlate with the calcium bioavailability (Kamchan, Puwastien, Sirichakwal, & Kongkachuichai, 2004). Our results also presented a significant negative correlation, between total oxalate and bioavailable calcium ($r = -0.35$). In this experiment, boiling the leaves had significant effect ($p < 0.01$) on the soluble oxalate content of the leaves. An earlier study reported losses of soluble oxalate between 38% and 87% from various vegetables such as spinach, Swiss chard and Brussels sprouts (Chai & Liebman, 2005). The losses of oxalate observed in the present study were less than those reported by Schonfeldt and Pretorius (2011) and more than those reported by Radek and Savage (2008), Gupta et al. (2006, 2005) and Kala and Prakash (2004).

The contents of oxalate in GLVs are shown in Table 2 and, as observed, boiling significantly decreased the oxalate content. In certain GLVs, there was a significant reduction ($p < 0.01$) in total oxalate due to cooking and the percentage loss ranged from 19.1% in *S. grandiflora* to 50.3% in *A. blitum* (Fig. S2; supporting information). Judprasong et al. (2006) found a significant loss of soluble oxalates from some Thai GLVs such as Cabbage, white stems and *Acacia pennata*.

Phytate content of cooked GLVs varied from 24 (*C. asiatica*) to 159.1 (*A. spinosus*) mg/100 g dry weight, respectively (Table 3). Cooking did not significantly change the phytate content of the GLVs. During cooking endogenous phytases are inactivated by heat. They are, therefore, unavailable to breakdown phytate, which can then only be degraded by high temperature processing (Yadav & Sehgal, 2003).

The cooking process did not significantly affect the tannin content of any of the selected GLVs. Tannin content of raw and cooked GLVs ranged between 86.4 and 84.4 mg/100 g in *C. album* and 424.7 and 416.1 in *S. grandiflora* (Table 3).

Amounts of SDF, IDF and TDF amount increased significantly ($p < 0.01$) in the cooked GLVs compared to the raw leaves (Fig. S3; supporting information), which is in line with the data presented by Kumari et al. (2004). Cooking caused a significant ($p < 0.01$) increase in the TDF content of almost all GLVs, probably due to hydration or polymerization of its fractions, as suggested by Mc Dougall, Morrison, Stewart, and Hillman (1996). SDF content increased significantly ($p < 0.01$) in cooked GLVs compared to raw samples (0.8–18.4%), confirming the results reported by Kumari et al. (2004). Cooking significantly ($p < 0.01$) increased IDF content for all GLVs studied (4.5–15.4%). The increase may be due to protein–fibre complexes formed after possible chemical modification induced by the cooking process (Bressani, 1993). These results are in agreement with the values obtained by Kumari et al. (2004).

The cooking process did not affect in vitro calcium bioavailability in any of the selected GLVs. If calcium bound to fibre cannot be absorbed in the small intestine, it may become available for absorption in the colon, where it can be released by the enzymatic action of microbiota, where non-digestible food component are fermented and short chain fatty acids produced (Ramirez-Moreno, Diez Marques, & Sanchez-Mata, 2011). Nevertheless, our results did not show any significant correlation between total fibre and calcium bioavailability, which is in agreement with other reports (Gupta et al., 2006; Kamchan et al., 2004). These reports found that some plant foods with low dietary fibre content, such as amaranth and wild betel, are low in bioavailable calcium, which may be due to the presence of other inhibitors in the food.

S. grandiflora, with the higher total Ca content, showed the lowest Ca solubility and bioavailability percentages (Table 1). The high oxalic acid content in *S.a grandiflora* (1199.2 mg/100 g; (Chawla, Saxena, & Seshadri, 1988) favours Ca precipitation and thus decreases its bioavailability. *Solanum nigram* also exhibited low calcium bioavailability due to high tannin content. *C. album*, *A. philoxeroides* and *C. asiatica* with lower total calcium content, had nearly twice as much bioavailable calcium than other GLVs, particularly *S. grandiflora*, *A. philoxeroides* and *P. alba*, because of their low fibre, oxalate, phytate and tannin content. The relationship between oxalate content and in vitro availability of calcium in selected GLVs in the present study is similar to that reported by Gupta et al. (2006) who observed that calcium availability in GLVs was lower because of high oxalate.

Among the four inhibitory factors, the amount of oxalate in plant foods was observed to have the greatest negative correlation

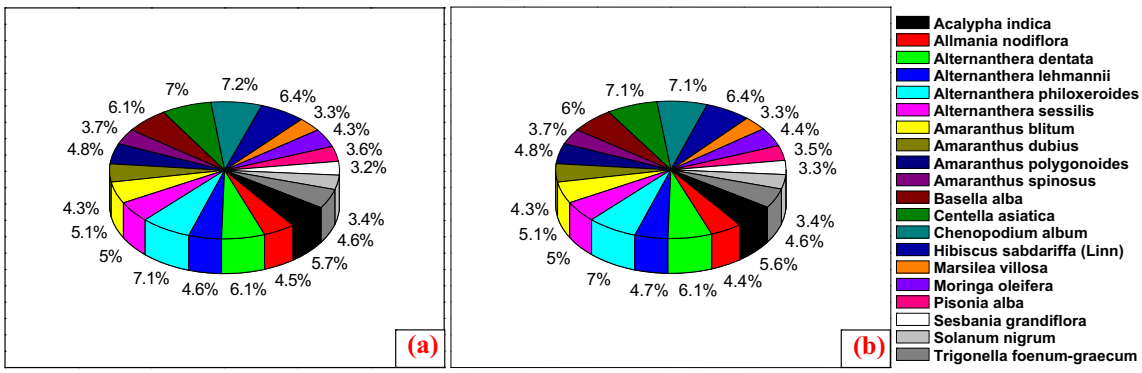

Fig. 2. Comparison of percentage of bioavailable calcium in (a) raw and (b) cooked GLVs.

with calcium bioavailability compared to tannin, phytate and dietary fibre. The percentage of calcium bioavailability was negatively correlated with the amount of inhibitory factors; the multiple regression analysis and correlation coefficients for soluble oxalate, tannin, total oxalate, phytate and SDF content were $r = -0.48^{*}$, -0.47^{*}, -0.33, -0.30 and -0.001 (Fig. 1) respectively in raw GLVs. In the case of cooked GLVs the percentage of calcium bioavailability was also negatively correlated with the amount of inhibitory factors; the r values for soluble oxalate, tannin, total oxalate, phytate and SDF are -0.48^{*}, -0.47^{*}, -0.33, -0.30 and -0.001 (Fig. 1) respectively ($^{*}p < 0.05$) (Table. S1; supporting information). The findings suggested that high contents of each of these inhibitory factors in both raw and cooked GLVs, especially oxalate and tannin, or in some combination could limit the bioavailability of calcium. Among the selected GLVs, *C. album*, *A. philoxeroides* and *C. asiatica* could be considered to have the most bioavailable calcium (Fig. 2).

4. Conclusions

This paper provides data on the content of total and bioavailable calcium as well as the levels of oxalate, phytate, tannin and dietary fibres of GLVs commonly consumed in India. The in vitro method employed for the estimation of calcium bioavailability is based on simulation of gastro-intestinal digestion. Calcium was an important constituent in all the GLVs examined, but bioavailability was limited due to the presence of oxalate, tannin, phytate and DFs. Calcium bioavailability and anti-nutritional factors were negatively correlated. Cooking did not significantly affect intestinal calcium bioavailability in the selected GLVs. *C. album*, *A. philoxeroides* and *C. asiatica,* with lower total calcium content, had nearly twice the bioavailable calcium than other GLVs, particularly *S. grandiflora*, *A. philoxeroides* and *P. alba*, because of their low fibre, oxalate, phytate and tannin content. The availability of calcium was found to be in the range of 14.8% and 15.3% in *S. grandiflora* to 32.7% and 33.1% in *C. album* in raw and cooked GLVs respectively. Our study revealed that oxalate is the highest inhibitory factor and dietary fibre is the lowest inhibitor for calcium absorption from GLVs. The consumption of GLVs, particularly *C. album*, *A. philoxeroides*, *C. asiatica*, *H. sabdariffa (Linn)*, *A. dentata*, *B. alba and A. indica* rich in bioavailable calcium, should be encouraged to reduce malnourishment as well as to prevent fluorosis by precipitating fluoride as insoluble calcium fluoride.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.08.031.

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